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(54) Title: GRP RECEPTOR-RELATED METHODS FOR TREATING AND PREVENTING FEAR-RELATED DISORDERS

(57) Abstract: This invention provides a method for treating a subject afflicted with a fear-related disorder comprising administering to the subject a therapeutically effective amount of a gastrinreleasing peptide receptor agonist. This invention further provides a method for inhibiting in a subject the onset of a fear-related disorder resulting from exposure to a traumatic experience comprising administering a prophylactically effective amount of a gastrin-releasing peptide receptor agonist to the subject prior to and/or following the traumatic experience. This invention further provides related articles of manufacture, and related nucleic acids and transgenic animals.

GRP RECEPTOR-RELATED METHODS FOR TREATING AND PREVENTING FEAR-RELATED DISORDERS

The invention disclosed herein was made with U.S. government support under grant number MH50733 from
5 the National Institute of Mental Health and with support under grant numbers NS44185 and DA15098 from the National Institutes of Health. Accordingly, the U.S. government has certain rights in this invention.

Throughout this application, various publications are
10 referenced by author and date. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these references in their entireties are hereby incorporated by reference into this
15 application to describe more fully the art to which this invention pertains.

Background of the Invention

Physiology of Fear

Fear is a basic, evolutionally conserved, emotion
20 which triggers a set of defensive mechanisms for adapting to threatening events that is essential for survival. A key component of the neural circuitry of fear, both innate and learned, in humans and in simpler vertebrate experimental animals is the
25 amygdala, a well-defined subcortical nuclear group (Davis and Whalen, 2001; LeDoux, 2000).

The memory of learned fear can be assessed quantitatively using a Pavlovian fear-conditioning paradigm (Fanselow and LeDoux, 1999; Kapp et al.,
30 1992). During fear conditioning, an initially neutral conditioned stimulus (CS) acquires biological

significance by becoming associated, following a few pairing trials, with an aversive unconditioned stimulus (US). After learning this association, the animal responds to the previously neutral CS with a

5   set of defensive behavioral response, which includes freezing, increased heart rate, and startle. The CS can be unimodal, involving only a single cure or modality such as a tone, light, smell, or touch. Alternatively, it can be multimodal, involving

10   several sensory modalities such as a context. Unimodal (cued) fear conditioning requires the amygdala but not the hippocampus. By contrast, multimodal (contextual) fear conditioning depends on both the hippocampus and the amygdala.

15   The lateral nucleus is the input region within the amygdala, where the association of learned information about CS and US occurs during auditory fear conditioning. The sensory information that mediates the CS – the auditory tone – reaches the

20   lateral nucleus by way of two neural pathways, both of which are essential for learned fear (Romanski and LeDoux, 1992). One pathway, the direct thalamo-amygdala pathway, originates in the medial geniculate nucleus (MGm) and in the posterior intralaminar

25   nucleus (PIN) of the thalamus. The second pathway, the indirect cortico-amygdala pathway, extends from the auditory thalamus to the auditory cortex (TE3 area) and includes a further projection that relays the processed auditory information from the cortex to

30   the lateral amygdala. After these two inputs are processed in the lateral nucleus, the signal is distributed to other amygdaloid nuclei (Pitkanen et al. 1997), including the central nucleus of the

amygdala (CeA), which projects in turn to areas in the brainstem that control autonomic (heart rate) and somatic motor centers (freezing) involved in the expression of fear.

5 Anatomical tracing and lesion studies first demonstrated the importance of the lateral nucleus for fear conditioning. Subsequent physiological experiments showed that learning produces prolonged synaptic modification in both of the inputs to the
10 lateral nucleus: the thalamo-amygdala pathway (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997) and the cortico-amygdala pathway (Tsvetkov et al., 2002). These synaptic modifications, which accompany behavioral learned fear, are
15 mechanistically similar to LTP induced artificially by electrical stimulation in tissue slices of the amygdala. By providing a direct causal link between slice LTP and memory storage, these studies establish the amygdala as perhaps the simplest and the best
20 model system in the mammalian brain for analyzing the cellular and molecular mechanisms of memory storage.

In contrast to the detailed cellular physiological information that is becoming available, the molecular machinery that underlies synaptic plasticity in
25 amygdala-dependent learned fear is largely unknown.

Neuropeptides and Anxiolytics

A number of neuropeptides are believed to be involved in the pathophysiology of anxiety, including, for example, cholecytokinin (CCK), corticotropin-
30 releasing factor and neuropeptide Y. Gastrin releasing peptide (GRP) is known as a potent satiety

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agent (see Merali, Z. et al. 1994). GRP antagonists
are also known in the field of cancer research for
their use in inhibiting tumor growth.

Gastrin releasing peptide (GRP) and neuromedin B
5   (NMB) are mammalian homologs of bombesin, a 14 amino
acid peptide hormone first isolated from the skin of
the frog, *Bombina bombina*. Three bombesin-like
peptide receptors are known: gastrin releasing
peptide receptor (GRPR; BB2), neuromedin B receptor
10   (NMBR; BB1) and bombesin receptor subtype-3 (BRS-3;
BB3). All are G-protein coupled receptors. Gastrin
releasing peptide receptor is known in the art by the
acronyms GRPR and BB2 (for bombesin recepotr subtype
2). Potent and selective peptide agonists of the
15   gastrin releasing peptide receptor (BB2) are known.
For example, Darker, J.G. et al. (2001) and Casibang,
M. and Moody, T.W. (2000) describe such agonists.
Bombesin agonists are also known, for example
Condamine E. et al. (1998).

20   More generally, assays for agonists or antagonists of
G-protein coupled receptors are known in the art.
See, for example, Fitzgerald, L.R., (1999).
Similarly, one of skill in the art can determine the
expression of GRPR in a cell or tissue sample using
25   routine methods (see Kusui et al. 1995).

Gamma-aminobutyric acid (GABA), along with
norepinephrine and serotonin, is known to be
important in the regulation of anxiety. GABA is the
major inhibitory neurotransmitter in the mammalian
30   central nervous system (CNS) and is utilized for
intercellular communication by approximately one-
third of all synapses in the CNS. There are two

classes of GABA receptors, A and B. The GABA-A receptor is comprised of five peptide subunits (alpha, beta, gamma, delta, and rho) which form a chloride-permeable ion channel coupled to a G-

5   protein. Each of the five subunits may have multiple isoforms. For example, there are six alpha, four beta, three gamma, one delta, and two rho subunits known presently.

Anxiolytics are compounds that relieve anxiety. Known

10   anxiolytic compounds include GABA-A agonists such as the benzodiazepines, which are the prototypic anti-anxiety compounds. Benzodiazepines interact with binding sites which are largely defined by the alpha subunit of the GABA-A receptor complex. In older

15   literature, the GABA-A receptor complex was referred to as the "benzodiazepine receptor" or BZR. More than two-dozen benzodiazepines are in clinical use in the United States. Among these are Alprzolam (Xanax), chlordiazepoxide (Librium), and diazepam (Valium).

20   Other examples of anxiolytic compounds are neurohormones such as 3-alpha, 5-alpha-pregnanolone (THPROG) and muscimol.

Animal tests for anxiolytic activity are known in the art. For example, one test involves pairing a reward

25   for which the animal must perform some behavior, such as lever pressing, with an aversive stimulus, such as mild electric shock. Agents that increase the rate of responses punished with the shock tend to be anxiolytic in humans (see basic Neurochemistry, 6th

30   ed. Siegel et al. editors). Another indicator of anxiolytic activity is a compound's binding affinity for the GABA-A receptor.

Summary of the Invention

This invention provides a method for treating a subject afflicted with a fear-related disorder comprising administering to the subject a
5   therapeutically effective amount of a gastrin-releasing peptide receptor agonist.

This invention further provides a method for inhibiting in a subject the onset of a fear-related disorder resulting from exposure to a traumatic
10 experience comprising administering a prophylactically effective amount of a gastrin-releasing peptide receptor agonist to the subject prior to and/or following the traumatic experience.

This invention further provides an article of
15 manufacture comprising (a) a packaging material having therein a gastrin-releasing peptide receptor agonist, and (b) a label indicating a use for the agonist in treating, and/or inhibiting the onset of, a fear-related disorder in a subject.

20 This invention further provides a nucleic acid comprising a gastrin-releasing peptide gene, wherein the gene has inserted into it, either at its start or stop codon, a polypeptide-encoding sequence, wherein the polypeptide is not gastrin-releasing peptide.

25

This invention further provides a transgenic animal whose somatic cells have stably integrated therein a nucleic acid comprising a gastrin-releasing peptide gene, wherein the gene has inserted into it, either
30 at its start or stop codon, a polypeptide-encoding sequence, wherein the polypeptide is not gastrin-

releasing peptide, and wherein the polypeptide is specifically expressed in the animal's amygdala.

Finally, this invention provides a method for
5 producing a transgenic animal whose amygdaloid cells specifically express an exogenous polypeptide, which method comprises producing a transgenic animal by introducing into an oocyte an exogenous DNA so that the exogenous DNA is stably integrated into the
10 oocyte, and permitting the resulting oocyte to mature into a viable animal, wherein (a) the animal's somatic cells have the exogenous DNA stably integrated therein, (b) the exogenous DNA comprises a gastrin-releasing peptide gene, wherein the gene has
15 inserted into it, either at its start or stop codon, an exogenous polypeptide-encoding sequence, and the exogenous polypeptide is not gastrin-releasing peptide, and (c) the exogenous polypeptide is specifically expressed in the animal's amygdala.

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Brief Description of the Figures

Figure 1: The Grp Gene is Specifically Expressed in the Lateral Nucleus/AB of the Amygdala and in the Cued and Contextual CS Pathways to the Amygdala.
5   Schematic of a mouse brain showing the location of coronal sections C1 and C2 and RNA in situ hybridization showing expression of the Grp gene therein. Below is a diagram depicting the major areas that send auditory and contextual information to the
10   amygdala obtained from tract-tracing studies.

Figure 2: GRP Receptors Are Functionally Expressed in Interneurons of the Lateral Nucleus of the Amygdala.

(A1) Bath application of GRP (200 nM) increased frequency of sIPSCs in a pyramidal cell from a
15   control mouse. The effect was blocked by 3uM bombesin antagonist (n = 6), thus suggesting that the GRP-induced enhancement of GABAergic tonic inhibition was specifically linked to the activation of the GRP receptors.

20   (A2) Effect of GRP on the frequency of sIPSCs is TTX-sensitive, and thus is dependent on action potential firing in interneurons.

(A3) GRP failed to increase the frequency of the picrotoxin-sensitive sIPSCs in GRPR knockout mice.

25   (B1) Representative sIPSCs recorded in a pyramidal cell from a control mouse at a holding potential of -70 mV under baseline conditions (left), during GRP application (center), and after the GRPR antagonist was added (right).

(B2) Representative sIPSCs recorded in a pyramidal
neuron from GRPR knockout mouse under baseline
conditions (left), during GRP application (center),
and after picrotoxin was added (right).

5   (C)   Cumulative   amplitude   histograms   of   sIPSCs
recorded under baseline conditions (filled symbols)
and after GRP was applied (open symbols) in slices
from control (left) and GRPR knockout mice.

Figure 3: Pairing-Induced LTP is Enhanced in GRPR
10   Knockout Mice. Pairing-induced LTP of whole-cell
EPSCs recorded in the lateral amygdala in wild-type
mice under control conditions (open symbols) and in
the presence of the bombesin antagonist (3 uM, filled
symbols).

15   (A)   A   schematic   representation   of   a   brain   slice
containing the amygdala that shows position of the
recording and stimulation pipettes.

(B)   LTP of whole-cell EPSCs recorded in the lateral
amygdala neuron in response to the cortical input
20   stimulation in slices from control (open symbols) or
GRPR knockout (filled symbols) mice. For induction of
LTP, the lateral amygdala neuron was held at +30 mV,
and 80 presynaptic stimuli were delivered at 2 Hz to
the external capsule fibers (arrow).

25   (C)   Current-voltage plot of the $GABA_A$ receptor IPSCs
at holding potentials of -110 mV to -10 mV. Reversal
potential of the IPSC mediated by the $GABA_A$ receptors
was -71 mV. Synaptic currents were recorded in the
presence of the AMPA receptor antagonist CNQX (20 uM)
30   and NMDA receptor antagonist D-APV (50 uM). Inset
shows $GABA_A$ receptor IPSCs recorded at holding

potentials of -110 mV to -10 mV. Traces are averages
of 10 IPSCs recorded at each holding potential.

(D)   Pairing-induced LTP of whole-cell EPSCs recorded
in the lateral amygdale in wild-type mice under
5   control conditions (open symbols) and in the presence
of the bombesin antagonist (3 uM, filled symbols).

Figure 4: GRPR-Deficient Mice Have Enhanced and
Resistant Long-Term But Not Short-Term Amygdala-
Dependent Fear Memory

10   (A1)   Contextual   fear   conditioning.   Significant
difference   in   freezing   responses   between   GRPR
knockout mice (n = 9, solid bars) and wild-type (n =
9, open bars) mice was found at 24 hr, 2, 7, and 15
weeks after training.

15   (A2) Cued fear conditioning. In response to the tone
(CS), both groups showed an increase in freezing.
However, this increase was significantly higher in
GRPR knockout animals, although no difference was
found between groups in the level of freezing before
20   the onset of the tone (pre-CS).

(B1) Contextual and (B2) cued-fear conditioning
assessed 30 min or 4 hr after training was normal in
GRPR knockout mice.

(C1-4) Water Maze. (wild-type, n = 9; knockout, n =
25   9). In this hippocampus-dependent memory task, both
groups of mice showed a similar rate of learning as
demonstrated by their equivalent latency (C1) to
reach the platform, whether it is during the visible
(Day 1 and 2) or hidden platform version of the task
30   (Day 3-6). They displayed the same swimming speed

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(C2), and thigmotaxis (% of time spent at the periphery; C3). They also showed equivalent performance in the probe trial (% of time spent in the different quadrant areas; C4), which assessed the

5   retention of spatially acquired information necessary to perform this task. GRPR knockout mice are no more sensitive or stressed than wild-type mice (D and E).

(D) Pain sensitivity thresholds. The intensity of shock required to elicit three reactions, movement

10   (movt), vocalization (vocal), and jump, was assessed and data are presented as the mean ± SEM. No difference was found between groups (wild-type, n = 10; knockout, n = 8).

(E) Elevated plus maze assessing basal anxiety. No

15   difference was found between GRPR (n = 18) and wild- type mice (n = 16) in the total number of entries, as in the number of entries in the closed or open arms.

Figure 5: A Model for GRP-Dependent Negative Feedback to Principal Neurons in the Amygdala in Wild-Type and

20   GRPR Knockout Mice.

Detailed Description of the Invention

Definitions

As used herein, "administering" shall mean delivering in a manner which is effected or performed using any
5   of the various methods and delivery systems known to those skilled in the art.   Administering can be performed, for example, intravenously, orally, via implant,   transmucosally,   transdermally, intramuscularly, or subcutaneously.   "Administering"
10 can also be performed, for example, once, a plurality of times, and/or over one or more extended periods.

As used herein, "agent" shall include, without limitation, an organic compound, a nucleic acid, a polypeptide, a lipid, and a carbohydrate.   Agents
15 include, for example, agents which are known with respect to structure and/or function, and those which are not known with respect to structure or function.

As used herein, "agonist of gastrin-releasing peptide receptor", or its synonymous term "gastrin-releasing
20 peptide receptor agonist", shall mean an agent that, when bound to gastrin-releasing peptide receptor, stimulates a biological response like the biological response stimulated when gastrin-releasing peptide is bound to gastrin-releasing peptide receptor.   For
25 example, an agonist of the GRP receptor can enhance inhibitory function of interneurons containing GRPR, which   leads   to   increased   GABA   release   by interneurons.   The   magnitude   of   the   biological response stimulated by a gastrin-releasing peptide
30 receptor agonist can be the same as, greater than or less   than   the   biological   response   stimulated   by

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gastrin-releasing peptide. Methods of identifying gastrin-releasing peptide receptor agonists are well-known in the art. (see e.g. U.S. Patent No. 5,741,651, at column 2, line 38). Gastrin-releasing

5 peptide agonists include, without limitation, bombesin, gastrin-releasing peptide fragments, and mutants of gastrin-releasing peptide and its fragments (e.g. point mutants), and analogs of gastrin-releasing peptide and its fragments (e.g.

10 gastrin-releasing peptide and its fragments wherein one or more amino acid residues are substituted with an amino acid derivative). Amino acid derivatives are well known in the art (see, e.g. U.S. Patent No. 6,552,061). Additional agonists are described in

15 Darker et al. 2001, and include, for example, GRP (aminoacids 19-27; available from Bachem, USA); [D-Phe^6, βALa^{11}, Phe^{13}, Nle^{14}]Bn(6-14) amide; [Glp^7, βALa^{11}, Phe^{13}, Nle^{14}]Bn(7-14) amide; [βALa^{11}, Phe^{13}, Nle^{14}]Bn(9-14) amide; and [βALa^{11}, Phe^{13}, Nle^{14}]Bn(10-

20 14) amide. These agonists have very high affinity to GRPR, but very little or no affinity to other receptors from the mammalian bombesin family.

As used herein, a "fear-related disorder" shall mean any disorder induced by or resulting from an event

25 that causes apprehension or alarm in the afflicted subject.

As used herein, "gastrin-releasing peptide" is a naturally occurring peptide that elicits gastrin release and regulates gastric acid secretion and

30 motor function in a subject. Gastrin-releasing peptide can be from a human or any other subject. The terms "gastrin releasing peptide", "gastrin-

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releasing peptide" and "GRP" are synonymous.

As used herein, with respect to claims for transgenic animals and methods of making same, "gastrin-releasing peptide gene", or "GRP gene", shall mean a
5   naturally occurring GRP-encoding DNA sequence (including introns), contiguous at its 5' end with at least about 30 kb of DNA sequence which is naturally contiguous with the 5' end of the GRP-encoding DNA sequence, and contiguous at its 3' end with at least
10   30kb of the DNA sequence which is naturally contiguous with the 3' end of the GRP-encoding DNA sequence.

As used herein, "inhibiting" the onset of a disorder shall mean either lessening the likelihood of the
15   disorder's onset, or preventing the onset of the disorder entirely. In the preferred embodiment, inhibiting the onset of a disorder means preventing its onset entirely.

As used herein, "nucleic acid" shall mean any nucleic
20   acid molecule, including, without limitation, DNA, RNA and hybrids thereof. The nucleic acid bases that form nucleic acid molecules can be the bases A, C, G, T and U, as well as derivatives thereof. Derivatives of these bases are well known in the art, and are
25   exemplified in PCR Systems, Reagents and Consumables (Perkin Elmer Catalogue 1996-1997, Roche Molecular Systems, Inc., Branchburg, New Jersey, USA).

As used herein, "protein" and "polypeptide" are used equivalently, and each shall mean a polymer of amino
30   acid residues. The amino acid residues can be naturally occurring or chemical analogues thereof.

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Polypeptides and proteins can also include modifications such as glycosylation, lipid attachment, sulfation, hydroxylation, and ADP-ribosylation.

5   As used herein, "subject" shall mean a human or any animal, such as a non-human primate, mouse, rat, guinea pig, dog, cat, or rabbit.

As used herein, a "traumatic experience" includes, without limitation, military combat, physical
10   assault, witnessing a physical assault, and experiencing a natural disaster, animal attack, or an emergency situation.

As used herein, "treating" a subject afflicted with a disorder shall mean either lessening the severity of
15   the disorder, or eliminating the disorder entirely.

Embodiments of the Invention

The present invention is based upon the discovery that gastrin-releasing peptide (GRP) is also an important regulator of certain types of anxiety,
20   namely those involving amygdala-dependent learned fear.

Specifically, this invention provides a method for treating a subject afflicted with a fear-related disorder comprising administering to the subject a
25   therapeutically effective amount of a gastrin-releasing peptide receptor agonist.

In one embodiment of the instant therapeutic method, the subject is human. Fear-related disorders treated include, for example, phobia, chronic anxiety, panic

attack, post-traumatic stress disorder, and autism.

This invention further provides a method for inhibiting in a subject the onset of a fear-related disorder resulting from exposure to a traumatic

5   experience comprising administering a prophylactically effective amount of a gastrin-releasing peptide receptor agonist to the subject prior to and/or following the traumatic experience.

In one embodiment of the instant prophylactic method,

10   the subject is human. An agonist would be administered, for example, prior to a foreseeable traumatic experience such as military combat. In one embodiment, the agonist would be administered between 1 and 20 days, or 5 and 10 days prior to the

15   traumatic experience. In a further embodiment, the agonist would be administered between 1 and 48 hours, or 12 and 24 hours prior to the traumatic experience. In a further embodiment, the agonist would be administered between 60 and 120 minutes, or 1 and 30

20   minutes prior to the traumatic experience.

An agonist would also be administered, for example, following a traumatic experience such as a physical assault. In one embodiment, the agonist would be administered between 1 and 20 days, or 5 and 10 days,

25   following the traumatic experience. In a further embodiment, the agonist would be administered between 1 and 48 hours, or 12 and 24 hours following the traumatic experience. In a further embodiment, the agonist would be administered between 60 and 120

30   minutes, or 1 and 30 minutes following the traumatic experience.

Determining a therapeutically or prophylactically
effective amount of the agonists used in the instant
invention can be done based on animal data using
routine computational methods. In one embodiment,
5   the therapeutically or prophylactically effective
amount contains between about 0.1 mg and about 1 g of
agonist. In another embodiment, the effective amount
contains between about 1 mg and about 100 mg of
agonist. In a further embodiment, the effective
10   amount contains between about 10 mg and about 50 mg
of agonist.

This invention further provides an article of
manufacture comprising (a) a packaging material
having therein a gastrin-releasing peptide receptor
15   agonist, and (b) a label indicating a use for the
agonist in treating, and/or inhibiting the onset of,
a fear-related disorder in a subject.

This invention further provides a nucleic acid
20   comprising a gastrin-releasing peptide gene, wherein
the gene has inserted into it, either at its start or
stop codon, a polypeptide-encoding sequence, wherein
the polypeptide is not gastrin-releasing peptide.
For mice, the GRP gene is located on chromosome 18.
25   The gene contains 3 exons, a transcript length of 862
bp, and a translation length of 146 residues. All
information regarding the GRP gene (coded by Q8R1I2
(SPTREMBL ID)) is contained at Ensemble site at
http://www.ensembl.org/Mus_musculus/geneview?gene=ENSMUSG0
30   0000024517.

This invention further provides a transgenic animal
whose somatic cells have stably integrated therein a

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nucleic acid comprising a gastrin-releasing peptide gene, wherein the gene has inserted into it, either at its start or stop codon, a polypeptide-encoding sequence, wherein the polypeptide is not gastrin-

5　　releasing peptide, and wherein the polypeptide is specifically expressed in the animal's amygdala. In one embodiment of the instant method, the transgenic animal is a mouse. Methods for making transgenic animals by introducing foreign DNA into embryonic

10　　cells and transplanting resulting cells into the uterus of an animal for development to term are well known in the art. (see e.g. U.S. Patent No. 4,870,009). The foreign DNA can be introduced either by gene targeting "knock-in" technology or BAC

15　　transgenic technology (using DNA recombination in bacteria).

Finally, this invention provides a method for producing a transgenic animal whose amygdaloid cells

20　　specifically express an exogenous polypeptide, which method comprises producing a transgenic animal by introducing into an oocyte an exogenous DNA so that the exogenous DNA is stably integrated into the oocyte, and permitting the resulting oocyte to mature

25　　into a viable animal, wherein (a) the animal's somatic cells have the exogenous DNA stably integrated therein, (b) the exogenous DNA comprises a gastrin-releasing peptide gene, wherein the gene has inserted into it, either at its start or stop codon,

30　　an exogenous polypeptide-encoding sequence, and the exogenous polypeptide is not gastrin-releasing peptide, and (c) the exogenous polypeptide is specifically expressed in the animal's amygdala.

This invention is illustrated in the Experimental
Details section which follows. This section is set
forth to aid in an understanding of the invention but
is not intended to, and should not be construed to,
5 limit in any way the invention as set forth in the
claims which follow thereafter.

Experimental Details

Synopsis

The present invention identifies two genes highly
enriched in the lateral nucleus of the amygdala: the
5   gastrin-releasing peptide (GRP) and oncoprotein 18
(Op18)/Stathmin. We focused on GRP because it is
presumably released as a cotransmitter with glutamate
in pyramidal cells of the lateral nucleus and its
receptor (GRPR) has been pinpointed as a candidate in
10   autism. This invention demonstrates that, when
released by activity, GRP acts on and excites
inhibitory interneurons by activating GRPR on their
cell surface. Activation of GRPR in these
interneurons in turn leads to an increase in the
15   level of tonic GABAergic inhibition in the principal
neurons. This invention further demonstrates that in
amygdala slices from GRPR knockout mice, the tonic
inhibition is markedly reduced and LTP is enhanced.
Consistent with this finding, these mice have
20   enhanced and prolonged long-term memory for fear to
both auditory and contextual cues, suggesting that
the GRP signaling pathways serves as an inhibitory
feedback constraint on learned fear.

Methods

25   *Animals*

GRPR knockout mice were described before and were
found grossly normal (Hampton et al., 1998). Mice
used for the study were back-crossed to N10 or more
to C57BL/6J strain.

Differential Screening

Amygdala cells were acutely dissociated as described
(Yu and Shin-nick-Gallagher, 1997). Cells
morphologically resembling pyramidal neurons were
5   identified under low magnification Nikon microscope
and individually transferred to PCR tubes containing
lysis buffer. cDNA libraries were synthesized as
described (Dulac and Axel, 1995). Five thousand
clones were differentially screened with the amygdala
10 and CA1 single cell cDNA probes. Amygdala probes for
the differential screening were enriched by two
rounds of subtraction of representational difference
analysis (Hubank and Schatz, 1994) against the CA1
cDNA.

15   *In Situ Hybridization/Immunohistochemistry*

Coronal sections from fresh-frozen mouse brains were
cut 20 microns thick and hybridized according to the
published protocol with modifications (Schaeren-
Wiemers and Gerfin-Moser, 1993). For dual fluorescent
20 in situ hybridization and immunohistochemistry,
digoxigenin-labeled RNA was first detected using
tyramide-based TSA Direct Fluorescein Kit (Perkin
Elmer). Then, sections were incubated with rabbit
antibody recognizing glutamic acid decarboxylase
25 (Chemicon) and detected using Cy3-conjugated donkey
anti-rabbit IgG (Jackson ImmunoResearch).

    Electrophysiology

Amygdala slices (250-300 um) were prepared from 3-5
week old control and GRPR knockout mice (littermates)
30 with a vibratome. Slices were continuously superfused
in solution containing (in mM): 119 NaCl, 2.5 KCl,

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2.5 $CaCl_2$, 1.0 $MgSO_4$, 1.25 NaH_2PO_4, 26.0 $NaHCO_3$, 10 glucose, and equilibrated with 95% O_2 and 5% CO_2 [pH 7.3-7.4] at room temperature. Whole-cell recordings of evoked compound EPSCs or spontaneous GABA-mediated

5   IPSCs were obtained from pyramidal cells in the lateral amygdala under visual guidance (DIG/ infrared optics) with an EPC-9 amplifier and Pulse v8.09 software (HEKA Elektronik). Compound EPSCs were evoked by stimulation of the fibers in the external

10   capsule at 0.05 Hz with a concentric stimulating electrode consisting of a patch pipette (10 um tip diameter) that was coated with silver paint (Bolshakov et al., 1997). The two leads of the stimulus isolation unit (ISO-Flex, Master-8

15   stimulator, AMPI, Jerusalem, Israel) were connected to the inside of the pipette and the external silver coat. The stimulating pipette was positioned to activate the cortical input to the lateral amygdala. To elicit the evoked $GABA_A$ IPSCs in the presence of

20   CNQX (20 uM) and D-APV (50 uM) in the bath, the stimulation electrode was placed within the lateral nucleus of the amygdala. The patch electrodes (3-5 MH resistance) contained (in mM): 120 KCl, 5 NaCl, 1 $MgCl_2$, 0.2 EGTA, 10 HEPES, 2 MgATP, and 0.1 NaGTP

25   (adjusted to pH 7.2 with KOH). In LTP experiments, 120 mM K-gluconate was used instead of KCI. To examine the voltage dependence of the evoked $GABA_A$ receptor IPSCs, cesium was substituted for potassium in the pipette solution. Series resistance was

30   monitored throughout experiment and was in a range of 10-20 milliohlms. Currents were filtered at 1 kHz and digitized at 5 kHz. The holding potential was -70 mV. In all LTP experiments, the stimulus intensity was adjusted to produce synaptic responses with an

amplitude which constitutes ~20%-25% of maximum
amplitude EPSC. Since we controlled for the size of
the baseline EPSC, the induction conditions were
identical for both LTP groups (control and knockout

5   mice). The EPSC amplitudes were measured as the
difference between the mean current during a
prestimulus baseline and the mean current over a 2
ms-window at the peak of the response. For induction
of LTP, 80 presynaptic stimuli were delivered at 2 Hz

10   to the external capsule fibers while the lateral
nucleus of the amygdala neuron was held at +30 mV for
the duration of the LTP-inducing presynaptic
stimulation. Summary LTP graphs were constructed by
normalizing data in 60 s epochs to the mean value of

15   the baseline EPSC.

The spontaneous IPSCs were recorded on videotape for
off line analysis in the presence of 20 uM CNQX. Data
were analyzed with the Mini Analysis Program v5.2.4
(Synaptosoft Inc., Decatur, GA; Bolshakov et al.,

20   2000).

    Behavior

For all behavioral tasks, mutant and control
littermates (males, 3 months old) were used.
Statistical analyses used ANOVAs with genotype as the

25   between subject factor, and session (fear
conditioning experiment), day, area (quadrant or
platform in the Morris water maze), or zone (elevated
plus maze and light-dark box) as within subject
factors. Mean ± SEM are presented. The experimenter

30   was blind to the genotype in all studies.

Fear conditioning experiments were done as described

(Bourt-chouladze et al., 1998). On the training day,
the mouse was placed in the conditioning chamber (Med
Associates) for 2 min before the onset of CS, a tone,
which lasted for 30 s at 2800 Hz, 85 dB. The last 2 s
5   of the CS was paired with US, 0.7 mA of continuous
foot shock. After an additional 30 s in the chamber,
the mouse was returned to its home cage. Conditioning
was assessed for 3 consecutive min in the chamber in
which the mice were trained by scoring freezing
10 behavior, which was defined as the complete lack of
movement, In intervals of 5 s. Mice (wild-type, n =
9; knockout, n = 9) were tested immediately after
training and at 24 hr, 2, 7, and 15 weeks after
training. For each time point, testing occurred first
15 in the context in which mice were trained (contextual
fear conditioning). Three hours after each contextual
testing session, mice were placed in a novel
environment (cued fear conditioning) in which the
tone (120 s) that has been presented during training
20 was given after a 1 min habituation period (pre-CS).

Pain Sensitivity Tests

Response to foot shocks was assessed with naive mice
(wild-type, n = 10; knockout, n = 8) as described
(Harrel, 2001). The intensity of shock required to
25 elicit running, vocalization, and a jump was
determined for each mouse by delivering a 1 s shock
every 30 s starting at 0.08 mA and increasing the
shock 0.02 mA each time. Testing was stopped after
all behaviors had been noted.

30   *Anxiety Tests*

.We performed two different tasks to assess basal

24

anxiety level in naive mice.

Elevated Plus Maze

The elevated plus maze consisted of a center platform and four arms placed 50 cm above the floor (Ramboz et
5 al., 1998). Two arms were enclosed within walls and the other two (open) had low rims. Naive mice (wild-type, n = 18; knockout, n = 16) were placed in the center and their behavior was recorded for 5 min with a camera located above the maze. Time spent (in
10 seconds, s) and entries in the different compartments (closed and open arms, center) were assessed.

Dark-Light Box

For the dark-light box test, mice (wild-type, n = 10; knockout, n = 9) were placed in the dark compartment
15 (head facing the wall) and observed for 5 min (Johansson et al., 2001). Time spent in and entries into the lit compartment were recorded.

Water Maze

The task was performed as previously described
20 (Malleret et al., 1999) with two training phases: 2 days with a visible platform followed by 4 days (spatial phase) with a hidden platform in the training quadrant (wild-type, n = 9; knockout, n = 9). For each phase, four trials, 120 s maximum and 15
25 min ITI (intertrial interval) were given daily. Probe trials (60 s), during which the platform was removed, were performed to assess retention of the previously acquired information.

Results

     Isolation of Genes Specifically Expressed in the
Lateral Nucleus of the Amygdala

     As an initial step in characterizing the molecular

5 mechanisms involved in learned fear, we searched for

genes enriched in the amygdala and, in particular, in

the lateral nucleus. To this end, we focused on

pyramidal projection neurons because these cells form

the majority of the constituent neurons in the

10 cortex-like nuclei of the amygdala to which the

lateral nucleus belongs and they transmit the CS and

US information during fear learning. We isolated

neurons using acute dissociation, which preserves

their processes and allows cell identification based

15 on neuronal morphology under the microscope (Yu and

Shinnick-Gallagher, 1997). Similarly, pyramidal

neurons were isolated from the anterior dorsal CA1

subregion of the hippocampus, which was chosen for

the comparison during cDNA library screening because

20 this region may be less involved in learned fear as

opposed to the ventral hippocampus (Bast et al.,

2001). We first used two rounds of representation

difference analysis (RDA) to enrich the lateral

nucleus cDNA probe against the CA1 cDNA sequences.

25 After differential screening of cDNA library derived

from single pyramidal amygdala neuron with probes

from the lateral nucleus and the CA1 region, we

analyzed candidate clones for gene expression pattern

using RNA in situ hybridization. We found two genes,

30 *Grp* and *Oncoprotein 18 (Op18)/Stathmin* expressed in

the lateral nucleus of the amygdala that had low or

no expression in the CA1 region of the hippocampus.

Interestingly, these two genes are also expressed in

the accessory basal nucleus (AB) of the amygdala, but
are absent in the basal lateral nucleus (BLA) that is
located between the lateral nucleus of the amygdala
and AB.

5   Both the *Grp* and *Op18/Stathmin* sequences originated
     from the screening of the same cell, which we
     identified as glutamatergic pyramidal neuron based on
     its shape during acute dissociation under the
     microscope and later by hybridizing its cDNA library
10   with different neuronal and glial markers and by
     subsequent characterization of the sequences
     comprising this cDNA library. This cDNA library (that
     contained the *Grp* and *Op18/Stathmin* sequences) was
     positive for neurofilament-L (NF-L, neuronal marker)
15   and it was negative for glial fibrilary acidic
     protein (GFAP, glial marker) and glutamic acid
     decarboxilase (GAD, interneuronal marker). In
     addition, we isolated from this library a cDNA that
     corresponds to the zinc transporter-3 (ZnT-3) gene, a
20   specific marker for zinc-containing subgroup of
     glutamatergic neurons, highly enriched in the limbic
     system and the lateral nucleus of the amygdala.

          *GRP Is Expressed in the Lateral Nucleus of the
          Amygdala and in the Regions Sending Synaptic
25   Projections to the Lateral Nucleus*

     Using in situ hybridization, we next found that the
     Grp gene is highly enriched in the lateral nucleus of
     the amygdala, and more specifically, in its dorsal
     and medial subnuclei. In addition, we observed strong
30   expression in the medial, ventral, and dorsal
     subdivisions of the medial geniculate body (MGm, MGv,
     and MGd), the posterior intralaminar nucleus (PIN) of
     the auditory thalamus, the TE3 subregion of the

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auditory cortex, and the perirhinal cortex (PRh, Figure 2B). All of these regions are afferently connected with the lateral nucleus of the amygdala and provide auditory inputs to the lateral nucleus of

5   the amygdala during fear learning (Pitkanen et al., 1997) suggesting that this peptide is involved in auditory cued fear conditioning. For example, MGm and PIN directly project auditory information to the lateral nucleus of the amygdala and to TE3. Area TE3

10   of the cortex in turn projects to the lateral nucleus of the amygdala (LeDoux, 2000). The ventral subiculum (VS), another structure where the *Grp* is localized, also provides a strong input to the medial division of the lateral nucleus of the amygdala as well as to

15   BLA and AB. PRh is reciprocally connected with the lateral nucleus of the amygdala and is capable of sending either cued or contextual signals. GRP is also expressed in the ventral dentate gyrus. However, a connection between the lateral nucleus of the

20   amygdala or AB and the dentate gyrus is not well documented.

GRPR Is Expressed in Inhibitory Interneurons

GRP is a peptide neurotransmitter that is selectively recognized by a seven transmembrane domain receptor

25   (GRPR) coupled to Gaq-protein (Hellmich et al., 1999). Having shown that GRP is expressed by principal cells in the lateral nucleus of the amygdala, we were curious to know what types of cells express GRPR. To identify the neurons within the

30   lateral nucleus of the amygdala that express GRPR, we performed colocalization studies using dual fluorescent in situ hybridization for *Grpr* RNA and immunohistochemistry with antibodies against

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interneuron-specific   marker,   glutamic   acid decarboxylase (GAD67 form, Figure 3A). We found that the *Grpr* RNA was expressed selectively in inhibitory GABAergic interneurons. However, GRPR was present

5   only in a subpopulation of GAD-positive interneurons, which suggests that the lateral nucleus of the amygdala contains various groups of interneurons subserving different functions.

Physiological, tract-tracing, and immunocytochemical

10   studies have shown that afferent signals converging onto the lateral nucleus of the amygdala are regulated locally in the dorsolateral division by inhibitory interneurons (Woodson et al., 2000). The afferent glutamatergic projections to the amygdala

15   synapse on both principal cells and GABAergic inhibitory interneurons (Mahanty and Sah, 1998). The inhibitory interneurons in turn send feedback inhibitory projections to pyramidal neurons. These feedback and feedforward GABAergic inputs are thought

20   to determine how the excitatory inputs to the principal cells involved in fear learning are processed and conveyed along neural pathways in the amygdala (Wang et al., 2001). The observed pattern of the *Grp* and *Grpr* genes expression suggested to us

25   that GRPR exerts a functional role in modulating the balance between excitation and inhibition in the local neuronal networks related to learned fear (see Figure 1).

30   *GRP Appears to Excite GABAergic Inhibitory Interneurons in the Lateral Nucleus of the Amygdala that Functionally Express GRPR*

To test whether activation of the GRP receptors on the GABAergic interneurons in the lateral nucleus of

the amygdala by the release of GRP from principal cells can change the level of tonic inhibition in the principal cell, we carried out whole-cell recordings from visually identified pyramidal neurons in mice.

5 We identified pyramidal neurons based on their appearance and their ability to demonstrate spike frequency adaptation to the prolonged depolarizing current injection (Tsvetkov et al., 2002).

We recorded spontaneous inhibitory postsynaptic

10 currents (sIPSCs) in the pyramidal neurons having blocked the AM PA (a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor-mediated responses (Figure 2) with CNQX (6-cyano-7-nitroquinoxaline-2,3-dione, 20 uM). To increase the inhibitory signals, we

15 inverted the inhibitory currents so that they had an inward direction by dialyzing the postsynaptic cells with a chloride-based intrapipette solution. Consistent with the notion that the sIPSCs are mediated by the $GABA_A$ receptors, these currents were

20 completely blocked (Figures $2A_3$ and $2B_2$) by gamma-aminobutyric acid-A ($GABA_A$) receptor antagonist, picrotoxin (50 uM, n = 10), at a holding potential of -70 mV.

In the absence of a $GABA_A$ receptor blockade,

25 application of GRP (200 nM) led to a significant increase in the frequency of sIPSCs in the soma of the principal cells of wild-type mice (baseline: 5.23 ± 0.68 Hz; GRP: 10.12 ± 1.0 Hz; n = 17 cells, obtained from 5 control mice; significant difference,

30 paired t test, t = 4.99, P < 0.0002; Figures 2A-2C). Therefore, we think that the increase in frequency of GABA sIPSCs was likely due to excitation of the interneurons by GRP leading to an increase in

30

the firing of action potentials in GABAergic
interneurons. We further supported this by blocking
the effects of the agonist by applying a Na^+ channel
blocker tetrodotoxin (TTX, 1 uM, n = 7; Figure $2A_2$).

5 These findings in the lateral amygdala are consistent
with previous work in the hippocampus, where
bombesin-like neuropeptides (including GRP) elicited
a marked increase in the frequency of $GABA_A$ receptor-
mediated IPSCs recorded in CA1 pyramidal neurons (Lee

10 et al., 1999) mediated by depolarization and induced
repetitive firing of GABAergic interneurons in the
stratum oriens.

We specifically linked the observed effect of the
bath-applied GRP to the activation of GRPR. Bath

15 application of a specific antagonist of GRPR ([D-
Phe^6,Des-Met^{14}]-bombesin-(6-14)ethyl amide; 3 uM; Lee
et al., 1999) blocked the effect of GRP on the
frequency of sIPSCs (Figures 2A, and 2B,; baseline:
5.15 ± 0.91 Hz; GRP: 10.37 ± 1.2 Hz; antagonist of

20 GRPR: 5.72 ± 1.1 Hz; n = 6 cells). The difference in
the frequency of sIPSCs in the baseline conditions
and after the GRPR antagonist application was not
statistically significant (paired t test, t = 1.21, P
= 0.3), suggesting that the bombesin antagonist fully

25 abolished the GRP-induced increase in the frequency
of the sIPSCs.

Knockout of GRPR Eliminates Tonic Inhibition

To obtain independent evidence that GRP induces en-
hancement of GABAergic tonic inhibition due to

30 activation of GRP receptors localized on
interneurons, we turned to mice in which the gene for
GRPR was knocked out. These mutant mice were

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littermates of the control mice we have studied to this point. GRPR knockout mice do not show any obvious developmental anatomical abnormalities throughout their body or their brain (Hampton et al.,
5   1998 and Figure 3B). Immunohistochemistry on brain sections of these mice with interneuron-specific antibodies (pan/albumin, calretinin, and calbindin) revealed no differences between knockout mice and wild-type controls. However, in situ hybridization
10   revealed that the GABAergic interneurons in the knockout mice were lacking GRPR. Consistent with these findings, we found in the mutants that the GRP-mediated negative control of the excitatory synaptic inputs to principal cells in the lateral nucleus was
15   lacking. In slices from mice in which the *Grpr* gene was ablated, bath-applied GRP failed to increase the frequency of sIPSCs (200 nM; baseline: 5.06 ± 0.58 Hz; GRP: 5.64 ± 0.67 Hz; n - 23 cells, obtained from 6 GRPR knockout mice; no significant difference:
20   paired t test, $t = 1.04$, $P = 0.31$; Figures $4A_3$, $4B_2$, and 4C). These results suggest that GRP receptors are functionally expressed in the lateral nucleus of wild-type mice and that activation of the GRP receptors on these interneurons was responsible for
25   the dramatic increase in the level of tonic GABA inhibition observed in the principal neurons in the lateral nucleus.

     LTP in the Cortico-Amygdala Pathway Is Enhanced in GRPR-Knockout Mice

30   Our recent findings indicate that LTP of the synaptic connections in the neural circuit of learned fear is an essential cellular mechanism contributing to the acquisition of memory for fear (Tsvetkov et al. 2002;

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see also McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997). Studies of different brain regions, including the hippocampus (Steele and Mauk, 1999), the cortex (Trepel and Racine, 2000), and the

5 amygdala (Rammes et al., 2000), indicate that modulation of principal cells by GABA-mediated inhibition can play an important role in the induction of LTP. We therefore asked: does removal of GRPR in the inhibitory interneurons affect LTP in

10 slices of the lateral nucleus of the amygdala?

We induced LTP of the compound glutamatergic EPSCs at the cortico-amygdala synapses by pairing postsynaptic depolarization from a holding potential of -70 mV to +30 mV with 80 presynaptic stimuli delivered to the

15 fibers in the external capsule (Huang and Kandel, 1998; Mahanty and Sah, 1998; Weisskopf and LeDoux, 1999) at a frequency of 2 Hz (Figures 3A-3B). We measured LTP with the K-gluconate containing intrapipette solution, without picrotoxin in the bath

20 (see Experimental Procedures). Under these experimental conditions, the peak amplitude of the evoked EPSC was solely determined by activation of the AMPA glutamate receptors. The contribution of the $GABA_A$ receptor-mediated component to the EPSC was

25 negligible at such holding potential since it was very close to the reversal potential (E_r) of $GABA_A$ IPSC $(-67 \pm 3$ mV, n = 6; Figure 3C). This induction protocol was used because, as we have shown previously, it consistently produces robust LTP

30 (Tsvetkov et al., 2002). We have deliberately chosen to depolarize a postsynaptic cell to a more positive membrane potential during the induction period, than in some previous studies, to allow a maximal

activation of L-type Ca^{2+} channels (e.g., Mermelstein
et al., 2000), as they were shown to take part in the
induction process (Tsvetkov et al., 2002; Weisskopf
et al., 1999). Keeping Ca^{2+} influx through L-type Ca^{2+}

5   channels at a relatively constant level, we minimize
a possible non-linearity of the interaction between
the   NMDA   receptor   and Ca^{2+} channel-mediated
contribution to the integral postsynaptic calcium
signal, thus maintaining the more uniform induction

10   conditions. When LTP at the cortical input to the
amygdala was compared (in a blinded fashion) in
slices from control and from GRPR knockout mice, we
found that LTP was significantly greater in knockout
than in control mice (Figure 3B), with an average LTP

15   of the EPSC to 2.02 ± 0.2 (n = 12 cells) and 1.33 ±
0.13 (n = 9 cells) of the baseline EPSC value,
respectively. The difference in the amount of LTP
measured over a 5 min period (between 35 and 40 min
after pairing) between control and knockout mice was

20   statistically significant (t-test, t = 2.96, P <
0.01). Thus, the ablation of the *Grpr* gene
disinhibits the pyramidal cells and makes the
cortico-amygdala synapses more susceptible to LTP. To
obtain independent support for this conclusion, we

25   measured the pairing-induced LTP in slices from wild-
type mice in the presence of the bombesin antagonist.
Under these conditions, LTP also was significantly
enhanced (Figure 5D, control LTP: 1.42 ± 0.04, n = 5
cells; LTP with the antagonist: 1.92 ± 0.05, n = 7

30   cells; significant difference, t test, *t* = 8.1, P <
0.0001).

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GRPR-Deficient Mice Have Enhanced and Persistent
Long-Term Memory for Fear to Both Auditory and
Contextual Cues

We first trained GRPR-deficient mice in Pavlovian
5   cued and contextual fear conditioning, an amygdala-
dependent task, which depends on the ability of the
animal to learn and remember that auditory cue or
context predict electric shock. During training, the
level of overall freezing of knockout animals was not
10   significantly different from wild-type littermate
controls. For both groups, freezing was slightly
increased within 30 s immediately after training
(Figure 4A). When tested for amygdala-dependent tone
fear conditioning, mice were placed in a new context
15   24 hr after training (Figure $4A_2$). Mice displayed an
increase in freezing at the onset of the tone (CS;
cued fear conditioning) as compared to the freezing
prior (pre-CS) to the tone (Session effect, all p <
0.01). In addition, the ANOVA revealed a significant
20   effect of genotype showing that GRPR knockout mice
froze   more   than   the   wild-type   mice   at   the
presentation of the tone which had been associated
previously with the electric shock (genotype effect:
$[F(1,16) = 13.30;$ p – 0.002]). Although freezing de-
25   creased with time in both groups of mice (Session
effect; all p < 0.01), GRPR knockout mice produced a
higher response to the tone in subsequent CS cued-
testing sessions at 2, 7, and 15 weeks (Genotype
effect; all p < 0.05).

30   Contextual fear conditioning is dependent both on the
amygdala and the hippocampus. Here, mice were tested
in the absence of cue in the same context 24 hr after
training. Both mutant and wild-type mice exhibited
higher level of freezing compared to immediately

after the shock (Session effect, all $p < 0.0001$, Figure 4A. This suggests that the mutant mice not only remembered the context where they received the shock the day before, but that they also developed

5 with time a strong aversive response to this environment associated with a painful experience. The ANOVA revealed a significant effect of genotype ($[F(1,16) = 25.07; p = 0.0001]$) showing that both groups of mice froze differently in this context,

10 with GRPR knockout mice showing a higher response as compared to their control littermates. Although freezing to context decreased in both groups of mice with time (Session effect, all $p < 0.0001$) suggesting similar rate of extinction, the observed increase in

15 freezing in GRPR knockout mice was still present in subsequent testing sessions at 2, 7, and 15 weeks (Genotype effect, all $p < 0.05$).

We also analyzed mutant mice for short-term memory at 30 min and at 4 hr in independent groups. For both

20 time points, there was no significant difference between mutant and wild-type mice in both contextual and cued fear conditioning (Figure 4B). Thus, the enhancement in learned fear observed in GRPR knockout mice is specific to long-term but not short-term

25 memory.

To verify that the increase in freezing displayed by GRPR knockout mice in the fear conditioning experiment was not due to an increased sensitivity to the shock, we performed a control experiment in which

30 we administered electric shock of increasing intensity while recording the behavioral response exhibited by the mice (Harrel, 2001). There was no difference between groups in the intensity of shock

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required to elicit movement, vocalization, or jump
(Figure 2D), indicating that an increase in freezing
observed in the fear conditioning experiments was due
to the learning process and not to a difference in
5   pain sensitivity.

        *The Enhanced and Persistent Fear Is Learned and
        Not Secondary to Chronic Anxiety*

To explore further these mice's tendency for innate
(not learned) fear, we used the elevated plus maze
10   where mice face a conflict between an innate aversion
to the open arms of the maze and the motivation to
explore this compartment (Ramboz et al., 1998). The
ANOVA conducted on the number of entries in the open
and closed arms and on the index of anxiety (time
15   spent/ entries in the open arms) did not reveal any
significant effect of genotype (Figure 4E). Thus, in
the elevated plus maze, the basal level of anxiety
was similar in control and GRPR knockout mice.

Another way to assess anxiety in mice is a light-dark
20   box test (Johansson et al., 2001). In this test, mice
tend to avoid the light compartment and naturally
prefer the dark one. Here again, we did not find any
difference between groups in the number of entries as
well as the total time spent in the lit compartment.
25   Thus, as with the elevated plus maze, the results
from the light-dark box test suggest that the basal
level of anxiety in GRPR knockout mice is similar to
that of wild-type mice.

        The GRPR Knockout Mice Show Normal Hippocampus-
30   *Dependent Spatial Memory*

Because GRP is expressed in the lateral nucleus of
the amygdala and specifically in its circuitry for

learned fear and we have found that knockout of GRPR
enhances amygdala-based learning, we were curious to
know if we can use GRPR-deficient mice to dissociate
amygdala-dependent   from   hippocampus-dependent

5 learning. To determine whether GRPR is important for
a purely hippocampus-based task, we turned to the
Morris water maze, a task in which the amygdala is
not involved. In this maze, an animal has to remember
the position of a hidden escape platform in

10 relationship to distal cues surrounding it in a
circular pool (Malleret et al., 1999). During
acquisition of the Morris water maze, mice from all
groups showed a decrease in escape latency (Figure
$4C_1$) across days, indicating learning of the platform

15 position (all groups, $p < 0.0001$). They also showed a
preference for the target quadrant during the probe
trial performed on the last day of the experiment
(Figure $4C_4$). We found no differences between groups
in this task (no genotype effect), suggesting that

20 the deletion of the GRPR does not enhance
hippocampus-dependent learning that is independent of
the amygdala, which is similar to the results of Wada
and coworkers (Wada et al., 1997). These results
support the notion that the amygdala is directly

25 involved in learned fear (Fanselow and LeDoux, 1999)
and that it does not merely modulate memories formed
in other brain structures like the hippocampus.

Discussion

We have identified, characterized, and localized to a
30 specific inhibitory neural circuit in the lateral
nucleus of the amygdala a molecular signaling network
important for learned fear. When this inhibitory
molecular   network   is disrupted,   mice   show

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increased LTP in the lateral nucleus of the amygdala
and an enhanced memory of learned fear as evident in
both cued and contextual fear conditioning. There is
a normal memory for hippocampus-based spatial task
5   indicating that this network is specifically involved
in the regulation of memory formation in the amygdala
in response to danger signals. There also is no
alteration in innate fear.

Experiments in humans and in experimental animals
10   over the last half a century indicate that the
amygdala is involved in learned fear (Davis and
Whalen, 2001; LeDoux, 2000). In the past 50 years, we
have learned a fair amount about the anatomy and cell
physiology underlying amygdala-based fear. For
15   example, recent experiments have demonstrated that
the mechanisms of LTP are recruited behaviorally at
the synapses in the lateral nucleus of the amygdala
during training for learned fear (Rogan et al., 1997;
McKernan and Shinnick-Gallagher, 1997; Tsvetkov et
20   al., 2002), thus providing direct support for the
link between LTP and memory storage. By contrast,
very little is known about the molecular mechanisms
contributing to this form of fear. This is
unfortunate because the neuronal pathways carrying
25   sensory information for unimodal learned fear (the
information carried by the CS) is much better
specified than that for the sensory information for
spatial learning as is the correlation between LTP
and memory storage.

30   We therefore have isolated amygdala-enriched genes
and then, using mouse genetics in combination with
physiological and behavioral approaches studied the
role of these genes in the memory for fear.

39

Initially, we isolated two genes expressed in a glutamatergic principal neuron of the lateral nucleus of the amygdala. The first of these genes, *Op18/Stathmin,* is highly expressed both in the

5 lateral nucleus of the amygdala and in the cerebral cortex with very little expression in the hippocampus. Op18/Stathmin is a phosphoprotein that binds tubulin dimers and destabilizes cellular microtubules (Belmont and Mitchison, 1996). It is a

10 major substrate for protein kinase A and upon phosphorylation releases tubulin thus allowing polymerization of tubulin molecules. *Op18/Stathmin* mRNA levels are increased after lesions to the perforant pathway of the hippocampus, which together

15 with the biochemical role of Op18/Stathmin protein suggests its involvement in synaptogenesis (Brauer et al., 2001).

The second gene, Grp, is uniquely localized in the lateral and accessory basal nuclei of the amygdala

20 and in regions that send projections to it and which are essential for delivering information about CS to the amygdala during Pavlovian fear conditioning (LeDoux, 2000). In particular, our analysis showed that the Grp gene is expressed both in the areas

25 specific to pathways delivering tone CS information and in the areas specific to pathways delivering contextual CS information. GRP is a 29 amino acid long mammalian homolog of the amphibian peptide bombesin (Kroog et al., 1995) and may serve as a

30 cotransmitter with glutamate in pyramidal neurons in the rodent brain (Lee et al., 1999 and our present data). Our observation of the Grp gene expression pattern specific to the fear network of the amygdala

finds support in the report that GRP concentration
was increased in the central nucleus of the rat
amygdala during both stress and feeding (Merali et
al., 1998). GRPR is a G q protein-coupled receptor

5   and its downstream targets include protein kinase C
(PKC-p) and phospholipase C as shown both in cultured
mouse fibroblasts and rat hippocampal neurons
(Mellmich et al., 1999; Lee et al., 1999). GRPR
activation by GRP binding leads to intracellular

10   release of Ca^{2+} and eventually to the activation of
the MAPK pathway (Sharif et al., 1997).

We found that GRP is expressed in a group of
glutamatergic principal neurons enriched in zinc.
Interestingly, zinc-containing glutamatergic neurons

15   constitute a specific network circuitry that includes
the lateral nucleus of the amygdala and other
components of the limbic system (reviewed in
Frederickson et al., 2000). We next found, as did Lee
and colleagues (1999), that GRPR is expressed in

20   GABAergic interneurons. We also found that GRPR
activation can significantly enhance the level of
tonic GABA-mediated inhibition in the lateral
nucleus. Recent pharmacological and genetic studies
have shown that the establishment of a balance

25   between glutamatergic excitatory and GABAergic
inhibitory functions is critical for processing of
information in the amygdala (Bast et al., 2001;
Krezel et al., 2001). Based on these published data
and our results, we suggest a model of GRP action in

30   the amygdala during fear response; during excitation,
the glutamatergic principal cells may release as a
cotransmitter the excitatory peptide, GRP. Through
the binding to GRPR on interneurons, GRP leads to

GABA release. This may provide tonic, feedforward, or
feedback inhibitory control of. the processing of CS
stimuli by principal cells (Figure 7, left image).
Thus, this molecular signaling pathway provides a

5   control which can regulate the balance between excit-
atory and inhibitory circuitries in the amygdala.

GRPR-Deficient Mice Show Both Enhanced LTP and
Enhanced Memory Storage in Amygdala-Dependent
Tasks

10 To test this model, we next examined the pyramidal
neurons in the lateral nucleus of the amygdala of
GRPR knockout animals and found that indeed they lack
an inhibitory control normally provided by GRP in
wild-type conditions (Figure 7, right image). As a

15 result of lacking inhibition, there is an enhanced
LTP in the cortico-amygdala pathway. In agreement
with our genetic finding, previous pharmacological
work has demonstrated that modulation of the level of
GABA-mediated inhibition of the principal cells in

20 the amygdala may determine how easily LTP is induced
at the amygdala synapses (Krezel et al., 2001; Rammes
et al., 2000).

Consistent with an enhancement of LTP, these animals
also show enhanced freezing in both cued and

25 contextual versions of amygdala-dependent fear
conditioning task. Throughout all time points tested
(the latest 15 weeks after training), mutant mice had
higher freezing than normal mice. This may be due to
faster fear memory retrieval in mutant mice because

30 during testing mutants started freezing right after
the tone was turned on but wild-types froze a few
seconds later. The fading over time of the phenotype
of GRPR knockout mice for fear conditioning might

reflect the contribution of shock-induced
sensitization in addition to the enhancement in
learning. In contrast to long-term effects, we found
that mutant mice have normal short-term memory when
5   tested at 30 min and even 4 hr after training. This
finding suggests the interesting possibility that
GRP/GRPR signaling pathway modulates learned fear in
a long-term specific manner and thus provides further
support to the notion that LTP is implicated in the
10 mechanisms of long-term memory. Importantly, GRPR-
deficient mice showed normal memory in the Morris
water maze, which is dependent on the hippocampus but
not the amygdala. This finding is again consistent
with fear circuitry-specific expression pattern of
15 the *Grp* gene and allowed us to dissociate amygdala-
based behavior from hippocampus-based behavior. Thus,
we identified a network that is specifically involved
in amygdala-dependent long-term memories for fear.

    A Possible Role of GRP Pathway in Mental
20   *Disorders*

The analysis of mice with decreased GABA function may
have important clinical implications. Decreased
levels of GABA have consistently been found in
patients with depression, panic, and generalized
25 anxiety disorders (Goddard et al., 2001) and some of
the drugs currently used to treat panic and
generalized anxiety disorders increase levels of GABA
in the brain (Parent et al., 2002). We did not find
any abnormalities in basal or in innate anxiety of
30 GRPR knockout mice probably because we did not
disrupt directly the biochemical machinery involved
in GABA production and utilization. Rather, we
interfered with the GABA functions by disrupting a

network that regulates GABA release. The reduction in
GABA release in mutant mice seems to fine-tune the
memory storage system so as to improve memory storage
for fear. Perhaps, greater depleting GABA would lead
5   to the opposite effects; it might decrease memory
storage for fear and lead to high levels of anxiety
similar to that described in mice mutant for GABA
receptors (Low et al., 2000; McKernan et al., 2000).
Since of all mental disorders anxiety disorders are
10   those that can best be modeled in mice and other
experimental animals (Bachevalier et al., 2001), it
is likely that molecular insights in the biology of
fear will prove to be broadly informative regarding
the genes important both for normal human fear and
15   for anxiety states.

Indeed, recent studies have suggested the possible
involvement of GRP and GRPR in mental disorders. *GRPR*
is a candidate gene for autism; an X;8 translocation
has been found that disrupted the first intron of the
20   *GRPR* gene in an autistic female patient (Ishikawa-
Brush et al., 1997). Importantly, genetic studies in
autistic patients have pinpointed chromosomal
abnormalities in the 15q11-q13, a region where the
GABRB3 gene is located, which codes for the beta 3
25   subunit of the gamma-amino-butyric acid (GABA)$_A$
receptor (Cook et al., 1998). Moreover, recent
behavioral, anatomical, and neuroimaging studies
suggest that one of the critical loci for autism
resides in the amygdala (Baron-Cohen et al., 2000).

30   Taken altogether, these observations demonstrate the
importance of determining molecular substrates of a

mygdala-dependent memory processes and identify the components of GRP/GRPR molecular network as a clear target for treating anxiety disorders.

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What is claimed is:

1. A method for treating a subject afflicted with a fear-related disorder comprising administering to the subject a therapeutically effective amount of a gastrin-releasing peptide receptor agonist.

2. The method of claim 1, wherein the subject is human.

3. The method of claim 1, wherein the fear-related disorder is a phobia.

4. The method of claim 1, wherein the fear-related disorder is chronic anxiety.

5. The method of claim 1, wherein the fear-related disorder is a panic attack.

6. The method of claim 1, wherein the fear-related disorder is post-traumatic stress disorder.

7. The method of claim 1, wherein the fear-related disorder is autism.

8. A method for inhibiting in a subject the onset of a fear-related disorder resulting from exposure to a traumatic experience comprising administering a prophylactically effective amount of a gastrin-releasing peptide receptor agonist to the subject prior to and/or following the traumatic experience.

9.   The method of claim 8, wherein the subject is
        human.

5   10.   The method of claim 8, wherein the fear-
        related disorder is a phobia.

        11.   The method of claim 8, wherein the fear-
        related disorder is chronic anxiety.

10

        12.   The method of claim 8, wherein the fear-
        related disorder is a panic attack.

        13.   The method of claim 8, wherein the fear-
15   related disorder is post-traumatic stress
        disorder.

        14.   The method of claim 8, wherein the agonist is
        administered to the subject prior to the
20   traumatic experience.

        15.   The method of claim 14, wherein the traumatic
        experience is military combat.

25   16.   The method of claim 8, wherein the agonist is
        administered to the subject after the
        traumatic experience.

        17.   The method of claim 16, wherein the traumatic
30   experience is a physical assault.

        18.   An article of manufacture comprising (a) a
        packaging material having therein a gastrin-
        releasing peptide receptor agonist, and (b) a

54

label indicating a use for the agonist in
treating, and/or inhibiting the onset of, a
fear-related disorder in a subject.

5   19.   A nucleic acid comprising a gastrin-releasing
peptide gene, wherein the gene has inserted
into it, either at its start or stop codon, a
polypeptide-encoding sequence, wherein the
polypeptide is not gastrin-releasing peptide.

10

20.   A bacterial artificial chromosome (BAC)
comprising the nucleic acid of claim 20.

21.   A transgenic animal whose somatic cells have
15   stably integrated therein a nucleic acid
comprising a gastrin-releasing peptide gene,
wherein the gene has inserted into it, either
at its start or stop codon, a polypeptide-
encoding sequence, wherein the polypeptide is
20   not gastrin-releasing peptide, and wherein
the polypeptide is specifically expressed in
the animal's amygdala.

22.   The transgenic animal of claim 22, wherein
25   the animal is a mouse.

23.   A method for producing a transgenic animal
whose amygdaloid cells specifically express
an exogenous polypeptide, which method
30   comprises producing a transgenic animal by
introducing into an oocyte an exogenous DNA
so that the exogenous DNA is stably
integrated into the oocyte, and permitting
the resulting oocyte to mature into a viable

55

animal, wherein (a) the animal's somatic
cells have the exogenous DNA stably
integrated therein, (b) the exogenous DNA
comprises a gastrin-releasing peptide gene,
5   wherein the gene has inserted into it, either
at its start or stop codon, an exogenous
polypeptide-encoding sequence, and the
exogenous polypeptide is not gastrin-
releasing peptide, and (c) the exogenous
10   polypeptide is specifically expressed in the
animal's amygdala.

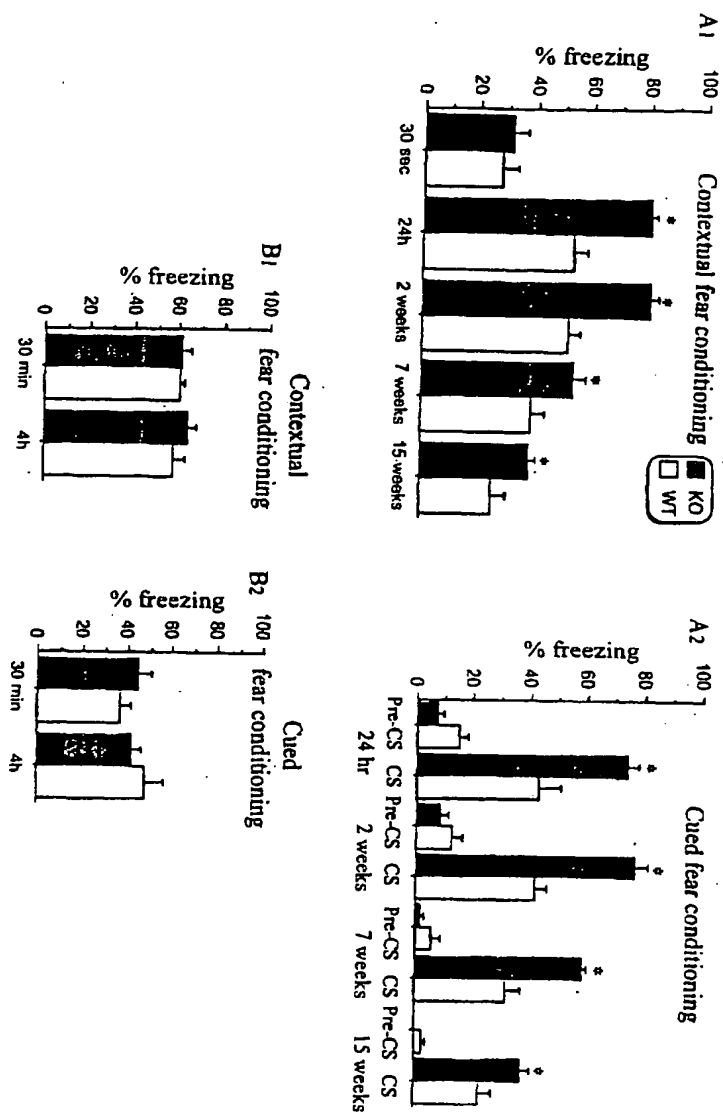24.   The method of claim 23, wherein the animal is
a mouse.

15
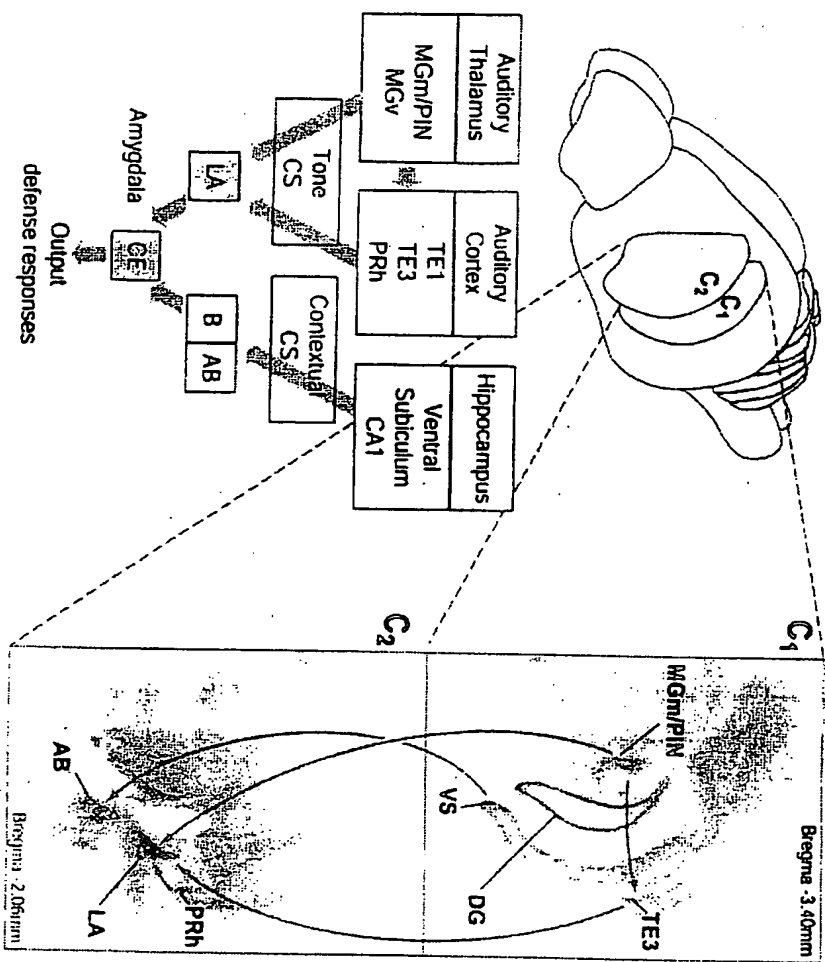
FIGURE 1
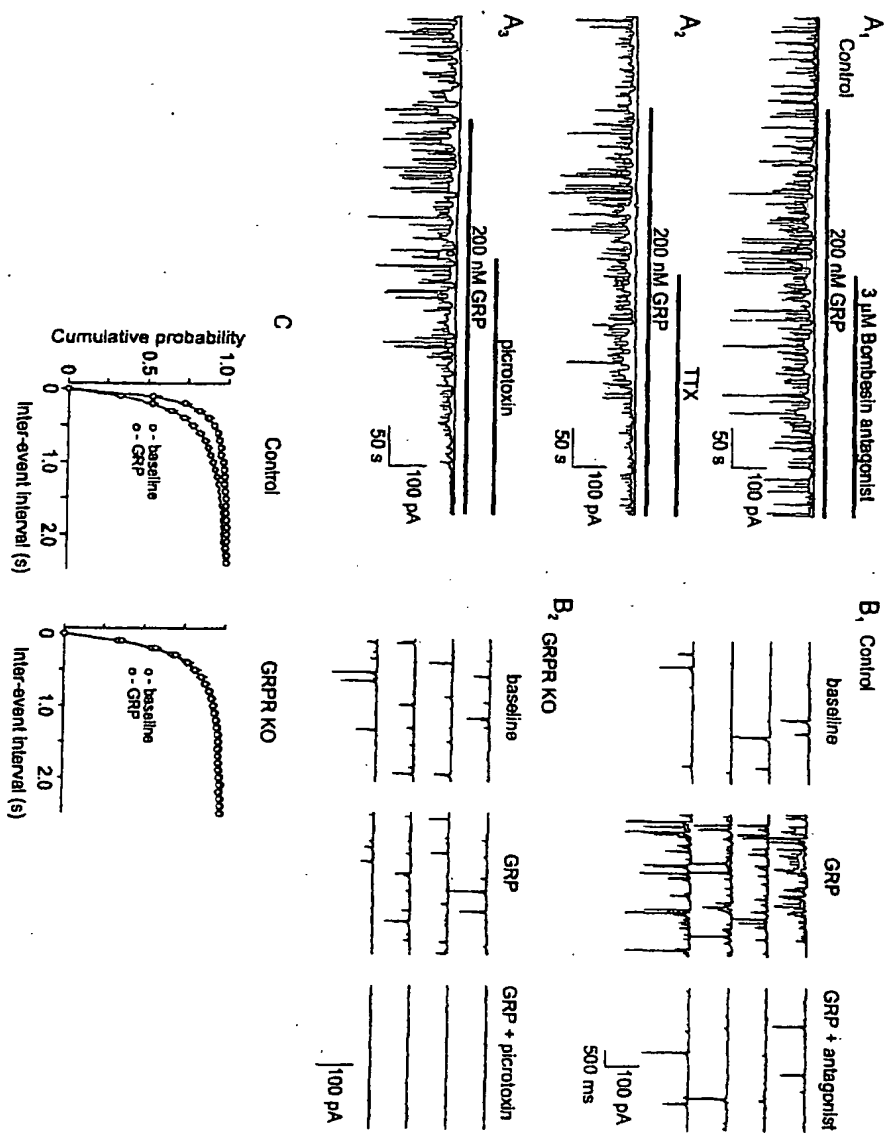
FIGURE 2
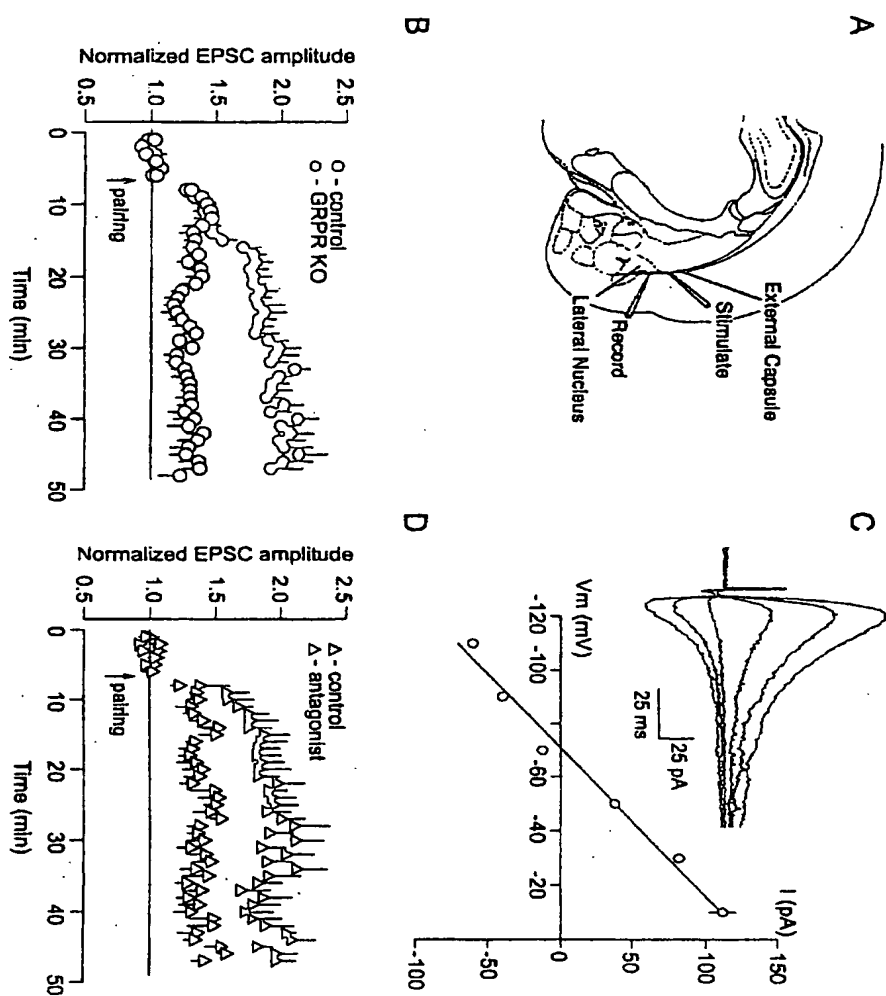
FIGURE 3

FIGURE 4 A-B

FIGURE 4 C-E

FIGURE 5